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Evaluation of higher plant virus resistance genes in the green alga, *Chlorella variabilis* NC64A, during the early phase of infection with *Paramecium bursaria* chlorella virus-1

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ABSTRACT

With growing industrial interest in algae plus their critical roles in aquatic systems, the need to understand the effects of algal pathogens is increasing. We examined a model algal host–virus system, *Chlorella variabilis* NC64A and virus, PBCV-1. *C. variabilis* encodes 375 homologs to genes involved in RNA silencing and in response to virus infection in higher plants. Illumina RNA-Seq data showed that 325 of these homologs were expressed in healthy and early PBCV-1 infected (≤60 min) cells. For each of the RNA silencing genes to which homologs were found, mRNA transcripts were detected in healthy and infected cells. *C. variabilis*, like higher plants, may employ certain RNA silencing pathways to defend itself against virus infection. To our knowledge this is the first examination of RNA silencing genes in algae beyond core proteins, and the first analysis of their transcription during virus infection.

Introduction

Algae are currently receiving considerable attention as a biofuel source. Certain eukaryotic algae (e.g., *Nannochloropsis* and *Chlorella* species) can produce fuel compatible lipids at a rate significantly higher than oil crop plants (e.g., soybean and palm) (Brennan and Owende, 2010). Additionally, certain algae can grow in regions that are unsuitable for typical crop plants and/or in brackish water. However, it is likely that algae grown at a large scale will experience pathogens, including viruses. Viruses are significant pathogens in traditional crop production and viruses may limit algal biomass yields. Furthermore, algal viruses are potentially a greater problem than higher plant viruses because many plant viruses are vectored by insects, mites, nematodes, or fungi. Thus, two components are required for a viral disease outbreak in higher plants; in contrast, no known vectors are required to spread algal viruses.

The field of algal virology is relatively new and only a few algal viruses have been extensively characterized (e.g., Brussaard and Martinez, 2008; Mackinder et al., 2009; Nagasaki, 2008; Van Etten and Dunigan, 2012; Wilson et al., 2009). Consequently, the complete range of their activities is unknown. However, given the global impact of algae, it is clear that algal viruses play a significant role in the environment (Brussaard et al., 2008; Jacquet et al., 2010; Suttle, 2007; Wilhelm and Matteson, 2008). Most of the research on algal viruses has been conducted on large dsDNA viruses that infect both freshwater and marine algae and are classified in the family *Phycodnaviridae*. ssRNA, dsRNA and ssDNA viruses have also been discovered in recent years that infect certain algae (Brussaard and Martinez, 2008; Nagasaki, 2008). Thus, a broad range of algae-infecting virus types exists in nature.

Viruses included in the *Phycodnaviridae* have genomes ranging from 160 to 560 kbp that contain up to 600 protein-encoding genes and many tRNA encoding genes (Van Etten et al., 2010; Wilson et al., 2009). One genus in the *Phycodnaviridae* consists of large icosahedral, plaque-forming viruses that infect unicellular, *Chlorella*-like green algae, genus *Chlorovirus*. Chloroviruses are present in freshwater environments throughout the world with titers as high as 100,000 plaque-forming units (PFU) ml^{-1} of indigenous water, although titers are typically in the range of 1–100 PFU ml^{-1} (Van Etten, 2003; Van Etten and Dunigan, 2012).

The prototype *Chlorovirus* is *Paramecium bursaria* chlorella virus-1 (PBCV-1), which infects *Chlorella variabilis* NC64A (formerly *Chlorella* NC64A). *C. variabilis* is an endosymbiont in the protozoan, *Paramecium bursaria*. *C. variabilis* can be grown independent of its partner in the laboratory, which allows plaque assays of the virus and synchronous infection of its host. These properties allow one to study the virus life cycle in some detail.

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Furthermore, both the PBCV-1 (Dunigan et al., 2012) and the *C. variabilis* (Blanc et al., 2010) genomes have been sequenced and annotated.

Previous studies on the initial steps of virus infection of *C. variabilis* established that PBCV-1 rapidly attaches to the host cell wall and degrades a portion of the cell wall at the point of attachment (Thiel et al., 2010; Van Etten and Dunigan, 2012). The viral membrane then presumably fuses with the host membrane, facilitating entry of the viral DNA and virion-associated proteins into the cell, leaving an empty capsid attached to the surface. PBCV-1 lacks a recognizable RNA polymerase gene and so circumstantial evidence suggests that the viral DNA quickly moves to the nucleus and steals the host transcription machinery. As reported here, certain early viral transcripts were detected by 7 min post infection (p. i.). Another early event that occurs during this time frame is that host chromosomal DNA begins to be degraded, presumably by PBCV-1 encoded and packaged DNA restriction endonucleases (Agarkova et al., 2006). The early stages of infection end when viral DNA synthesis begins, ~60–90 min p.i. (Meints et al., 1986). Therefore, if the host cell is to initiate a response to virus infection it must do so within this early timeframe.

Although substantial information is available on the viral events associated with PBCV-1 infection (e.g. Thiel et al., 2010; Van Etten and Dunigan, 2012), very little is known about the initial response(s) of the host cell to virus infection. A related phycodnavirus, EhV-86, that infects the marine alga, *Emiliania huxleyi*, inhibits the host cell's programmed cell death (PCD) mechanism, which insures that the viruses have time to replicate (Bidle et al., 2007). Although *E. huxleyi*'s attempt to avoid EhV-86 replication through PCD ultimately results in cell death, it is clear that *E. huxleyi* has a means of resisting virus infection. The direct observations of developed resistance, the presence of viral genes in algal host genomes, and the apparent coevolution of algae and viruses in nature (Blanc et al., 2010; Derelle et al., 2008; Lindell et al., 2004; Monier et al., 2009; Thomas et al., 2011) all suggest that algae have mechanisms to avoid and/or limit virus infection.

Green algae and land plants share a common ancestry (Lewis and McCourt, 2004; Parfrey et al., 2010), and in higher land plants, the responses to virus infection, and to pathogens in general, have been studied extensively (e.g. Bari and Jones, 2009; Chisholm et al., 2006; Katiyar-Agarwal and Jin, 2010; Nimchuk et al., 2003; Soosaar et al., 2005). One mechanism, RNA silencing, active in both plants and animals, has the potential to be an important means of survival against virus infection in algae, particularly green algae (Cerutti and Casas-Mollano, 2006; Whitham et al., 2006). Presently RNA silencing has been best studied in the green alga *Chlamydomonas reinhardtii* (Cerutti et al., 2011; Schroda, 2006). However, no viruses have been found that infect this model alga, limiting its use for studying algal host–virus interactions. *C. variabilis* and PBCV-1 provide an ideal pair for examining the presence of RNA silencing genes in a host and their expression during virus infection. Despite *C. variabilis* being a competent host for PBCV-1 infection, the initial response of *C. variabilis* to infection might provide important information about a potential innate immune response to virus infection in these algae.

Arabidopsis is a well-studied model for understanding higher plant defenses, including RNA silencing (Aufsatz et al., 2002; Brodersen and Voinnet, 2006; Hammond et al., 2001; Matzke et al., 2009; Whitham et al., 2006). Key genes in the RNA silencing pathways such as argonauts (AGOs), dicer-like proteins (DCLs), and RNA dependent RNA polymerases (RdRPs) have been searched for in the genomes of 20 eukaryotic algae, covering a wide taxonomic range, including nine green algae (Cerutti et al., 2011). Though the presence of homologs to AGOs and DCLs suggests that RNA silencing may occur in *C. variabilis*, RNA silencing requires several other participants. In this manuscript we compile a more complete list of genes potentially involved in RNA silencing in *C. variabilis* by searching for homologs to those known to be involved in RNA silencing in *Arabidopsis*. We also searched for homologs to other genes known to be induced by virus infection in *Arabidopsis* (Whitham et al., 2006). Furthermore, to discern whether or not *C. variabilis* utilizes these genes, we examined their expression in uninfected cells and in cells that were infected with PBCV-1 for 7, 14, 20, 40, and 60 min using RNA-seq Illumina data.

Results and discussion

Higher plant virus resistance genes in C. variabilis

Thirty-four RNA silencing genes that have been identified in higher plants, primarily *Arabidopsis* (plus two additions: helicases and ribonuclease family III members), and an additional 27 genes that are involved in response to virus infection in *Arabidopsis* (Brodersen and Voinnet, 2006; Matzke et al., 2009; Whitham et al., 2006) were used as queries against the *C. variabilis* genome using BLASTp. For these 61 genes and two general gene categories (see Table 1), 375 homologs were present in the *C. variabilis* genome, including 14 putative *Arabidopsis-C. variabilis* ortholog pairs identified by the reciprocal best BLASTp hit criterion (Table S1). Of the detected genes, 219 are homologs to those induced upon virus infection in *Arabidopsis*, while the remaining 156 are homologs to those involved in RNA silencing, with 73 of these being helicases and four possible DCLs classified as ribonuclease family III members (i.e. the two gene categories). Table S1 lists all of the genes searched for in the genome of *C. variabilis* and the corresponding list of homologs found along with the e-values supporting their similarity to the query sequences, putative orthologous genes are also noted.

While *C. variabilis* had homologs to many of the genes of interest, not all were detected using one or more *Arabidopsis* queries or even motif searches using MEME; 19 of the 61 were not detected. This indicates that either these genes are absent in *C. variabilis*, or are so divergent that they are not recognized by these methods. The fact that we found homologs to many of the genes of interest supports the practicality of using information gathered from higher plant studies to begin examining similar processes in green algae.

How many of the 375 putative resistance genes are expressed during PBCV-1 infection?

The RNA-Seq approach (Mortazavi et al., 2008) produces millions of short cDNA reads that are mapped to a reference genome to obtain a genome-scale transcriptional map, which consists of the transcriptional structure and the expression level for each gene. We used the Illumina RNA-Seq method to gain insight into the transcriptional responses that are associated with infection of *C. variabilis* by PBCV-1. More than 105 million sequence reads, 50 bp in length, were generated from six time points during the first hour of infection – i.e., 0, 7, 14, 20, 40 and 60 min p.i. The sequence reads were aligned onto the *C. variabilis* genome and then analyzed to measure gene expression levels. We used this data to analyze the changes in transcription activity of the 375 resistance genes during PBCV-1 infection. The global results of the RNA-Seq study will be presented in a separate paper (Rowe et al., in preparation). Briefly, of *C. variabilis*'s 9792 genes, 16 had no read mapped to them at any point during infection suggesting that they were not expressed under the conditions of the study. An additional 1867 had read numbers below 50 at any point and were designated as weakly expressed. Of the remaining 7909 genes, 5335 did not display any differential expression ≤2-fold change during infection after read count normalization. The

Table 1
List of gene groups examined in the genome and transcriptomes of *C. variabilis*. Transcriptomes were of healthy/uninfected cells ($T=0$) and of cells infected with PBCV-1 for up to 60 minutes. Loci listed are of *Arabidopsis* genes used to search *C. variabilis*'s genome. Numbers in the "Homologs" column reflect the number of homologs found in the *C. variabilis* genome. When no homologs were detected in *C. variabilis*, we noted the results of searching all the currently sequenced genomes of other green algae. Numbers in the "Expressed" column reflect the number of homologs detected at all time points. Numbers provided in the "Up- or Down-Regulation" column reflect only those homologs whose expression changed 2-fold or greater, as compared to $T=0$ expression (the absence of a number indicates no change in expression ≥2-fold).

Protein	TAIR locus	Homologs	Expressed	Up- or Down-regulation	Description	References
RNA silencing present in *C. variabilis* genome						
ArGOnaute 1 and 4 (AGO1 and 4)	AT1G48410 and AT2G27040	1	1	1 Up	Work within the RISC, bind to siRNAs and miRNAs, involved in RdDM and PTGS	1
ChromoMeThylase 3 (CMT3)	AT1G69770	4	3		Involved in CNG and CNN methylation, main methylase involved in non-CG methylation maintenance, likely controlled by SUVH4, SUVH5, SUVH6, and possible more in Arabidopsis	2
CLaSsY1 (CLSY1)	AT3G42670	18 that overlap with DRD1	17 that overlap with DRD1	2 Up	SNF2-like chromatin remodeling protein, required for proper localization of RDR2, possible role in a step between NRPD1 and RDR2 to produce siRNAs and spread transgene silencing	3
DiCer-Like 1, 2, 3, and 4 (DCL1–4)	AT1G01040, AT3G03300, AT3G43920, and AT5G20320	1	1		Produce 21–24 nt RNAs from longer substrates, differ based on specificity of substrates and cleavage sites	4
Defective in RNA-directed DNA methylation 1 (DRD1)	AT2G16390	21	19	3 Up	Putative SWI2/SNF2-like chromatin remodeling protein, essential for RdDM, works with Pol V to signal DRM2, needed for adding and removing methylation in response to the presence and absence, respectively, of RNA signals	5
Domains Rearranged Methylase 2 (DRM2)	AT5G14620	1	1		Involved in CG, CNG, and CNN methylation, major *de novo* DNA methyltransferase in RdDM, signaled by Pol V, along with DRM1 likely works with MET1 for full *de novo* CG methylation	6
EXoRiboNuclease 4 (XRN4)	AT1G54490	2	2		5'-3' exoribonuclease, involved in ethylene response, can work to suppress PTGS, affects siRNA and miRNA decay, degrades and allows accumulation of certain transcripts based on sequence and functional category	7
HASTY	AT3G05040	1	1		Member of importin/exportin family, involved in miRNA transport out of nucleus	8
Helicase	n/a	73	70	8 Up / 9 Down	As a domain within dicers may act to unwind siRNA precursor, as a group of proteins functions in all aspects of RNA metabolism, may also function in protein degradation, some are required for RNA silencing	9
Histone Deacetylase 6	AT5G63110	9	7	2 Down	1 of 2 histone modifying enzymes within RdDM, removes acetyle groups from lysine residues in N-termini of histones, reinforces CG methylation induced by RNA, physically interacts with MET1, may play additional roles in silencing not mediated by RNA, may provide free histone lysines to KYP for DNA methylation	10
Hua ENhancer 2 (HEN2)	AT2G06990	11	10	3 Up	Putative DExH-box RNA helicase, involved in RNA metabolism, likely involved in degradation of improperly sliced mRNAs	11
KrYPtonite (KYP) aka SUVH4	AT5G13960	6	6	1 Down	A histone H3K9 methyltransferease, 1 of 2 histone modifying enzymes within RdDM, maintains cytosine methylation primarily in CNGs, may receive free histone lysines from HDA6 for DNA methylation	12
KU70	AT1G16970	1	1		Forms a heterodimer complex with KU80, interacts with and stimulates WEX's exonuclease activity, may also be involved in repairing dsDNA breaks	13
KU80	AT1G48050	1	1	1 Up	Forms a heterodimer complex with KU70, interacts with and stimulates WEX's exonuclease activity, may also be involved in repairing dsDNA breaks	14
METhyltransferase 1 (MET1)	AT5G49160	2+4 that overlap with CMT3	2+3 that overlap with CMT3	1 Up (without overlap)	Maintains CG methylation, physically interacts with HDA6, likely works with DRM1 and DRM2 for full *de novo* CG methylation	15
Nuclear RNA Polymerase D2 (NRPD2) aka NRPD2A or DRD2 or NRPE2	AT3G23780	3	3		Second largest subunit of Pol IV and Pol V (a catalytic subunit), involved in producing and amplifying siRNAs when part of Pol IV, involved in signaling *de novo* methylation when part of Pol V and joining with DRD1	16
Nuclear RNA Polymerase E5 (NRPE5)	AT3G57080	2	1		A non-catalytic subunit of Pol V, involved in signaling *de novo* methylation when part of Pol V and joining with DRD1	17

Table 1 (*continued*)

Protein	TAIR locus	Homologs	Expressed	Up- or Down-regulation	Description	References
Ribonuclease Family III	n/a	4	4	3 Up	Cleave dsRNA into different size classes of RNA, include DCL (4 classes) and non-DCL members	18
Silencing DEfective 3 (SDE3)	AT1G05460	7	6	2 Up / 1 Down	An SF1 family member, may remove secondary structures in target transcripts, may amplify secondary siRNAs for transmitting PTGS signal, in *Drosophila* is required for RISC assembly	19
SErrate (SE)	AT2G27100	1	1		Physically interacts with DCL1 and HYL1 in miRNA pathway to process pri-miRNAs, helps to stabilize pri-miRNAs	20
SUpressor of Variegation 3–9 Homolog protein 2 (SUVH2)	AT2G33290	4 that overlap with KYP	4 that overlap with KYP	1 Down	Possess H3K9, H3K27, and HK20 methylation activity	21
SUpressor of Variegation 3–9 Homolog protein 5 (SUVH5)	AT2G35160	1+6 that overlap with KYP and 1 with SUVR3	1+6 that overlap with KYP and 1 with SUVR3	1 Down (overlaps with KYP)	Helps maintain H3K9 methylation, may control CMT3, with KYP controls transposons	22
SUpressor of Variegation 3–9 Related protein 3 (SUVR3)	AT3G03750	2+3 that overlap with KRY	2+3 that overlap with KRY		Histone-lysine N-methyltransferase, involved in histone-lysine and peptidyl-lysine methylation	23
Werner-like EXonuclease (WEX)	AT4G13870	2	2	1 Up / 1 Down	DEDD superfamily of 3'-5' exoribonucleases member, forms a complex with KU70/80 heterodimer which stimulates exonuclease activity, required for PTGS	24
RNA Silencing absent from *C. variabilis* genome						
Domains Rearranged Methylase 1 (DRM1)	AT5G15380	Not present			Involved in CG, CNG, and CNN methylation, major *de novo* DNA methyltransferase in RdDM, signaled by Pol V, along with DRM2 likely works with MET1 for full *de novo* CG methylation	25
Hua ENhancer 1 (HEN1)	AT4G20910	Not present			Methylates siRNAs and miRNAs at the 3' end to protect from uridylation, works after DCLs and before AGO1 in PTGS pathways	26
HYponastic Leaves 1 (HYL1)	AT1G09700	Not present			Nuclear dsRNA binding protein, physically interacts with DCL1, HYL1, and SE in miRNA pathway to process pri-miRNAs, likely aids in correct positioning of DCL1	27
Nuclear RNA Polymerase D 1 (NRPD1) aka SDE4 or NRPD1a	AT1G63020	Present in all (generally 3 per genome)			1 of 2 alternative largest subunits of Pol IV, involved in producing and amplifying siRNAs when part of Pol IV	28
Nuclear RNA Polymerase E 1 (NRPE1) aka DRD3 or NRPE1 or NRPD1b	AT2G40030	(Same protein family as above)			Largest subunit of Pol V, involved in signaling *de novo* methylation when part of Pol V and joining with DRD1	29
Nuclear RNA Polymerase D4/E4 (NRPD4/NRPE4)	AT4G15950	Not present			A non-catalytic subunit of Pols IV and V, involved in producing and amplifying siRNAs when part of Pol IV, involved in signaling *de novo* methylation when part of Pol V and joining with DRD1	30
Nuclear RNA Polymerase D7 (NRPD7)	AT3G22900	Not present			A non-catalytic subunit of Pol IV, involved in producing and amplifying siRNAs when part of Pol IV	31
Nuclear RNA Polymerase E7 (NRPE7)	AT4G14660	(Same protein family as above)			A non-catalytic subunit of Pol V, involved in signaling *de novo* methylation when part of Pol V and joining with DRD1	32
RNA Dependent RNA Polymerase 2 (RDR2)	AT4G11130	Not present			Important for RdDM, critical (with DCL3) for production of certain siRNAs, not required for tasiRNAs or miRNAs production	33
RNA Dependent RNA Polymerase 6 (RDR6)	AT3G49500	Only 1 ortholog in *Coccomyxa* sp. C-169 (homologous to RDR2).			Works with SGS3 to produce dsRNA for DCL4 to cleave into 21nt siRNAs, may depend on SGS3	34
Suppressor of Gene Silencing 3 (SGS3)	AT5G23570	Not present			Binds and protects cleaved transcript from degradation prior to RDR6 action, interacts with RDR6	35
SUpressor of Variegation 3–9 Homolog protein 6 (SUVH6)	AT2G22740	Present in most (including *C. variabilis*)[a]			Helps maintain H3K9 methylation, may control CMT3, with KYP controls dsRNA from transcribed inverted repeats	36
Virus induced (in *Arabidopsis*) present						

Table 1 (*continued*)

Protein	TAIR locus	Homologs	Expressed	Up- or Down-regulation	Description	References
in *C. variabilis* genome						
Aldo-Keto reductase	AT2G37770	11	8	1 Down	An NADPH-dependent aldo-keto reductase, accepts a wide range of substrates, primary role may be to detoxify stress-related ketones and aldehydes, upregulated under different stresses including infection	37
Alpha/Beta-hydrolases Superfamily	AT2G39420	2	1	1 Down	Members share same α/β-sheet with 8 strands connected by helices fold, family consists of cellular and secreted proteins, larger ones found in eukaryotes, in plants smaller ones tend to be soluble	38
Calmodulin 2, 5, and Calmodulin-like MSS3	AT2G41110, AT2G27030, AT2G43290	16	11	3 Up	Signal various pathways including stress responses, activate and inactive proteins via Ca^{2+} binding	39
Calreticulin 3	AT1G08450	2	2	2 Down	Involved in signaling via Ca^{2+} binding, known to be localized to the ER with PDIs but may have an extra-ER location, may act as chaperones	40
Chitinase, Class IV and putative	AT3G54420 and AT2G43570	1	1		Pathogenesis-related 3 protein family, involved in and possibly involved in defense against fungi, nematodes, and insects, may act to loosen cell walls	41
Cytochrome P450	AT2G45570	12	9	5 Up	Has e^- carrier and monooxygenase activities, involved in response to phosphate and water limitation, expressed during the hypersensitive response and various processes leading to cell death	42
Glucose-6-phosphate Dehydrogenase 2 and 3	AT5G13110 and AT1G24280	2	2		Rate-limiting enzyme within the pentose phosphate pathway, provides major source of NADPH in the cell	43
Glutathione S-transferase 16 and Phi 16	AT2G02930 and AT4G02520	1	1		Within phi class of glutatione s-transferases, specific to plants, involved in defense response to fungi, regulation controlled by multiple mechanisms	44
Heat Shock Protein 70	AT3G12580 and AT5G02490	12	12	1 Up / 4 Down	Central to network of chaperones and folding catalysts, assists refolding of non-native proteins, prevents aggregations, involved in responses to heat, bacteria, viruses, and increases in unfolded proteins in the ER lumen	45
Heat Shock Protein 83	AT5G52640	5	5		Involved in protein folding, trafficking, and degradation, signal transduction, cell cycle control, and in defense response to bacteria, possibly involved in stress adaptation in *Arabidopsis*	46
Pathogenesis-related Gene 1	AT2G14610	3	1		Pathogenesis-related 1 protein family, involved in defense activity against oomycetes, activated by chemical treatment, attack by insect, or infection	47
Polyubiquitin 4 and 10	AT5G20620 and AT5G05320	10	8	2 Up	Contains multiple, linked ubiquitin coding regions, involved in protein degradation	48
Protein Disulfide Isomerase (PDI)	AT3G54960	8	8		Forms and breaks disulfide bonds, involved in metabolism, protein folding, cellular redox homeostasis, and in the response to endoplasmic reticulum stress	49
Protein Phosphatase 2C	AT4G08260 and AT3G27140	8	7	1 Up / 2 Down	Within group B of protein phophatases M family within protein serine/threoine phosphatases class, likely has high sustrate specificity, strongly induced by stress	50
Putative Protein Kinase	AT2G31880, and AT5G13290	97	79	15 Up / 3 Down	Involved in regulation of multiple cell death and plant resistance signaling pathways via phosphorylation	51
Senescence-related Gene 1 (SRG1)	AT1G17020	6	5	1 Up	Member of the Fe(II)/ascorbate oxidase superfamily, oxidoreductase activity, may be involved in cell wall modification in abscission zones	52
SUMO-activating Enzyme 1A (SAE1A)	AT4G24940	4	4	1 Up	Necessary for catalysis, involved in sumolation, responds to abiotic stresses, may be directly involved in plant pathogenesis	53
Thioredoxin H-type 1, 3, 4, and 5	AT3G51030, AT5G42980, AT1G19730, and AT1G45145	13+2 that overlap with PDI and 1 with Calmodulin 2	11+2 that overlap with PDI and 1 with Calmodulin 2	1 Up / 4 Down (without overlap)	All belong to subgroup 1, reduce disulfide bonds in other proteins, reduced by NADPH mediated by NADPH-reductase, may be involved in protection against oxidative stress and in regulating redox-dependent signaling cascades, may be involved in nitrogen and carbon metabolism and in self-incompatibility	54
Tyrosine Aminotransferase 3	AT2G24850	5	5		Converts tyrosine to *p*-hydroxyphenylpyruvate, involved in production of radical scavengers, induced by jasmonate, methyl jasmonate, coronatine, methyl-12-oxophytodienoic acid, and wounding	55
WRKY 6	AT1G62300	1	1	1 Up	A key regulator of plant innate immunity, involved in senescence, response to herbivory and bacteria, boron and phosphate limitation, and chitin	56

Table 1 (*continued*)

Protein	TAIR locus	Homologs	Expressed	Up- or Down-regulation	Description	References
Virus induced (in *Arabidopsis*) absent from *C. variabilis* genome						
Beta-1,3-glucanase	AT3G57260	Not present			Member of pathogenesis-related 2 protein family, may aid in generating signaling molecules for downstream defense mechanisms, may degrade microbial cell walls, may block virus transport	57
Copper/Zinc Superoxide Dismutase Copper Chaperone	AT1G12520	Orthologs in *Ostreococcus* and *Micromonas* spp.			Assists in Cu incorporation and catalysis of disulfide bond formation to lead to the activation of copper/zinc superoxide dismutase	58
Glutamate Receptor 2.7	AT2G29120	Not present			Member of putative ligand-gated ion channel subunit family, involved in ion transport, homeostasis of Ca^{2+}, systemic acquired resistance, and in the responses to light and ER stress	59
Metalloproteinase	AT1G24140	Not present			Binds Zn, has metalloendopeptidase and peptidase activity, involved in ER unfolded protein response	60
Pathogenesis-related Gene 5/Thaumatin-like	AT1G75040	Not present			Pathogenesis-related 5 protein family, may function in signal transduction, associated with antifungal activity	61
Pectin Methylesterase	AT1G11580	Not present			Catalyzes pectin deesterification, shows ribosome-inactivating protein activity, involved in cell wall modification	62
Senescence-associated Gene 21 (SAG21)	AT4G02380	Not present			Involved in response to and tolerance of reactive oxygen species, induced by dehydration and ethylene treatment	63

References 1=Brodersen and Voinnet (2006), Höck and Meister (2008), 2=Brodersen and Voinnet (2006); Ebbs and Bender (2006), Huettel et al. (2007), Lindroth et al. (2001), 3=Smith et al. (2007), 4=Olmedo and Guzman (2008), 5=Huettel et al. (2007), 6=Aufsatz et al. (2004), Huettel et al. (2007); Singh et al. (2008), 7=Gregory et al. (2008), Rymarquis et al. (2011), Souret et al. (2004), Swarbreck et al. (2007), 8=Brodersen and Voinnet (2006), Park et al. (2005), Swarbreck et al. (2007), 9=Boisvert and Simard (2008), Linder and Owttrim (2009), Olmedo and Guzman (2008), 10=Huettel et al. (2007), Kim et al. (2012), 11=Jackson et al. (2002); Linder and Owttrim (2009); Swarbreck et al. (2007); Western et al. (2002), 12=Brodersen and Voinnet (2006), Huettel et al. (2007); Jackson et al. (2004), 13=Downs and Jackson (2004); Li et al. (2005), Lieber et al. (2003); Swarbreck et al. (2007), 14=Downs and Jackson (2004); Li et al. (2005); Lieber et al. (2003); Swarbreck et al. (2007), 15=Aufsatz et al. (2004); Huettel et al. (2007), Kim et al. (2012), 16=Huettel et al. (2007); Matzke et al. (2009), 17=Matzke et al. (2009); Swarbreck et al. (2007), 18=Olmedo and Guzman (2008), 19=Brodersen and Voinnet (2006); Linder and Owttrim (2009), 20=Swarbreck et al. (2007); Xie et al. (2010), 21=Naumann et al. (2005), 22=Ebbs et al. (2005); Ebbs and Bender (2006); Jackson et al. (2004), 23=Swarbreck et al. (2007), 24=Brodersen and Voinnet (2006); Glazov et al. (2003); Swarbreck et al. (2007), 25=Aufsatz et al. (2004); Huettel et al. (2007); Singh et al. (2008); Swarbreck et al. (2007), 26=Brodersen and Voinnet (2006); Jack (2002); Swarbreck et al. (2007), 27=Brodersen and Voinnet (2006), Swarbreck et al. (2007), Xie et al. (2010), 28=Huettel et al. (2007), Matzke et al. (2009), Swarbreck et al. (2007), 29=Huettel et al. (2007), Matzke et al. (2009); Swarbreck et al. (2007), 30=Matzke et al. (2009); Swarbreck et al. (2007), 31=Matzke et al. (2009), Swarbreck et al. (2007), 32=Matzke et al. (2009), Swarbreck et al. (2007), 33=Kurihara et al. (2008); Lu et al. (2006); Xie et al. (2004), 34=Béclin et al. (2002); Kumakura et al. (2009), 35=Béclin et al. (2002), Kumakura et al. (2009), 36=Ebbs et al. (2005), Ebbs and Bender (2006), Jackson et al. (2004), 37=Simpson et al. (2009), 38=Koschorreck et al. (2005); Swarbreck et al. (2007), 39=Reddy et al. (2011), 40=Crofts and Denecke (1998), 41=Van Loon et al. (2006), 42=Godiard et al. (1998), Swarbreck et al. (2007), 43=Stanton (2012), Swarbreck et al. (2007), 44=Swarbreck et al. (2007); Wagner et al. (2002), 45=Aparicio et al. (2005), Huang and Xu (2008), Noël et al. (2007), Swarbreck et al. (2007), 46=Huang and Xu (2008); Swarbreck et al. (2007), Takahashi et al. (1992), 47=Van Loon et al. (2006), 48=Swarbreck et al. (2007), 49=Swarbreck et al. (2007), 50=Schweighofer et al. (2004), 51=Gao et al. (2009); Swarbreck et al. (2007), 52=Callard et al. (1996), Swarbreck et al. (2007), 53=Kurepa et al. (2003), Miura et al. (2007); Saracco et al. (2007), 54=Gelhaye et al. (2004), Swarbreck et al. (2007), 55=Sandorf and Holländer-Czytoko (2002), 56=Robatzek and Somssich (2001), Rushton et al. (2010), Swarbreck et al. (2007), 57=Van Loon et al. (2006), 58=Brown et al. (2004); Casareno et al. (1998); Furukawa et al. (2004), Huang et al. (2012), Lamb et al. (2001), 59=Swarbreck et al. (2007), 60=Swarbreck et al. (2007), 61=Van Loon et al. (2006), 62=De-la-Peña et al. (2008), and 63=Miller et al. (1999), Swarbreck et al. (2007)

[a] In this study, we disqualified SUVH6 homologs found in *C. variabilis* based on lack of SUVH6-specific domains and greater homology of these sequences to KYP, SUVH2, SUVR3, and SUVH5 homologs.

remaining 2574 genes exhibited ≥2-fold changes with approximately half showing up-regulation and half showing down-regulation.

Table 2 summarizes the homologs detected in the genome and transcriptomes of *C. variabilis*. Of the 375 genes of interest coded by *C. variabilis*, 325 were expressed in healthy cells and during the first 60 min of infection. Transcripts for another 48 genes were detected, but at low levels (<50 mapped reads) and were not included in our counts of expressed genes, two more had no reads at any point. Of the 325 expressed genes, 85 had expression levels that changed ≥2-fold during the course of the infection. A hierarchical clustering analysis revealed two broad expression patterns (Fig. 1): within the 85 variable genes, 31 were globally down-regulated, while the remaining 54 were up-regulated genes. Table S2 lists all 375 genes of interest and their normalized read counts over the six time points of the infection.

RNA silencing

One process that is expected to be utilized for virus resistance by *C. variabilis* is RNA silencing. RNA silencing serves a variety of biological purposes aside from defense against virus infections. However, it appears that viral defense is an ancestral function (Cerutti and Casas-Mollano, 2006). Because of the ancestral development of RNA silencing, the critical role it can play in defense against viruses in higher plants, and the evolutionary connection between green algae and higher plants, we looked for RNA silencing genes in the *C. variabilis* genome that were homologous to those studied in *Arabidopsis* (Brodersen and Voinnet, 2006; Matzke et al., 2009). There are several pathways that fall under the term RNA silencing, which in plants can be grouped under either RNA-directed DNA methylation (RdDM) or post-transcriptional gene silencing (PTGS) (Aufsatz et al., 2002;

Table 2
Summary of *Arabidopsis* genes used as queries against the *C. variabilis* genome, homologs detected in the *C. variabilis* genome, homologs expressed, and homologs that undergo 2-fold or greater changes during infection.

Gene class	Number of *Arabidopsis* genes	Number of *C. variabilis* homologs of *Arabidopsis* genes	Expressed in healthy *C. variabilis*	Number of *C. variabilis* genes with 2-fold difference in expression during infection
RNA silencing	34+2 gene categories	156	145	23 Up-regulated 14 Down-regulated
Virus induced (in *Arabidopsis*)	27	219	180	31 Up-regulated 17 Down-regulated

Brodersen and Voinnet, 2006; Ghildiyal and Zamore, 2009; Hammond et al., 2001; Matzke et al., 2009).

The different RNA silencing pathways are not mutually exclusive and share several key proteins: e.g., dicers or dicer-like proteins (DCLs), argonauts (AGOs), and RNA-dependent RNA polymerases (RdRPs) (Cerutti and Casas-Mollano, 2006; Höck and Meister, 2008). Figs. 2–6 present simplified schematics of our current understanding of these pathways. It should be noted that RdRPs are not critical for RNA silencing in all organisms. One pertinent example is another green alga, *Chlamydomonas reinhardtii*, which like *C. variabilis*, lacks a recognizable RdRP. However, RNA silencing has been demonstrated in *C. reinhardtii* as well as other organisms that lack RdRPs (Cerutti and Casas-Mollano, 2006). Of the 36 genes and gene categories we examined, homologs for 24 were present in *C. variabilis*, including nine putative orthologs (AGO1, DCL1, MET1, HASTY, HEN2, SUVR3, KU70, KU80, and WEX), and of the 156 homologs detected, 145 were expressed. It should be noted that the pathway positions of a number of genes involved in RNA silencing are not known.

C. variabilis has one putative ortholog each of a DCL (homologous to DCLs 1–4, orthologous to DCL1) and an AGO (homologous to AGOs 1 and 4, orthologous to AGO1), both of which were expressed in uninfected cells and in the first 60 min of PBCV-1 infection. The AGO was >2-fold up-regulated. Four additional genes had high similarity with DCLs, however they lacked some of the domains required to classify them as DCLs. Consequently, we categorized them under the broader term as members of the ribonuclease family III. This family of proteins cleaves dsRNA into different size classes of RNA and contains both DCL and non-DCL members; the structures of the latter are simpler than those of DCLs and the functions of only a few are currently known (Olmedo and Guzman, 2008). DCLs are highly variable in regards to their amino acid sequences and the organization of their domains. Moreover, the DCLs of some organisms, whose RNA silencing activity has been confirmed, do not contain all the domains of a typical dicer (Cerutti and Casas-Mollano, 2006). Because of this, we included the four ribonuclease family III members in our examination. All four of these were expressed during infection, with three up-regulated. It is possible that these ribonucleases function as dicers for *C. variabilis*. It should be noted that PBCV-1 also encodes a functional ribonuclease III gene that is expressed very early in virus infection (Zhang et al., 2003).

Additionally, DCLs have a helicase domain. Although this domain is not always required and its specific function is unknown, it is predicted to unwind the precursor to small interfering RNAs (siRNAs) (Boisvert and Simard, 2008; Olmedo and Guzman, 2008). As proteins, helicases can function in all aspects of RNA metabolism, including roles in RNA silencing (Linder and Owttrim, 2009). Therefore, we also examined 73 helicases present in the *C. variabilis* genome. Seventy of these helicases were expressed, eight of which were up-regulated and nine of which were down-regulated.

RNA directed DNA methylation

In RNA-directed DNA methylation (RdDM), Pol IV (NRPD2 +NRPD1+NRPD4/E4+NRPD7) generates ssRNA transcripts from targeted DNA that may be slightly methylated or possibly from nascent RNA. These transcripts are converted into dsRNA by the RNA dependent RNA polymerase, RDR2. CLSY1, which may work in the nucleus with Pol IV, is required for proper localization of RDR2. The dsRNA is further processed into 24 nt siRNAs by DCL3, which can then further direct the cleavage of nascent transcripts. These transcripts also may guide proteins such as HDA6, which physically interacts with MET1, SUVH2, and KYP, which together with SUVH5 and SUVH6 likely control CMT3 (which is also involved). DRD1, along with Pol V (NRPD2+NRPE1+NRPD4/E4+NRPE5 +NRPE7), which physically interacts with AGO4, signals *de novo* methylation of DNA via DRM2 (Brodersen and Voinnet, 2006; Ebbs and Bender, 2006; Huettel et al., 2007; Matzke et al., 2009) (see Fig. 2).

During infection, *C. variabilis* expressed the three NRPD2 subunits of Pol IV and Pol V that are present in the genome along with one of the two NRPE5 subunits of Pol V that are also present. The remaining subunits of both Pol IV and Pol V were not identified in the genome. RDR2 was not present in the genome, but 17 of the 18 CLSY1 genes (homology shared with DRD1) were expressed, two of which are up-regulated. Seven of the nine HDA6s (two down-regulated) were expressed. Both MET1 genes (those without similarity to CMT3) were expressed, with one up-regulated. All six KYPs and all four SUVH2s (which share homology with each other) were expressed, one of which was down-regulated during infection. The one SUVH5 (without similarity to KYP) and three of the four CMT3s were expressed without change over the first 60 min of infection. SUVH6 was not present in the genome. Nineteen of the 21 DRD1 genes were expressed. Two of these do not share homology with CLSY1 and one of these was up-regulated along with the two mentioned earlier. Lastly, the one DRM2 was expressed.

For those homologs not found in the genome, *C. variabilis* might use other proteins for the same function. NRPD2 joins with several subunits to form Pol IV and Pol V, respectively. It is curious that all three NRPD2 homologs were expressed, despite only one other subunit (specific to Pol V) being present in the genome. If the missing subunits are vital to Pol IV and/or Pol V function, then what is the purpose in expending the energy to make a portion of either polymerase? Moreover, nearly all other participants in this pathway were present and expressed. Though certainly not definitive, this suggests that a Pol V-like protein, may be functioning in the cell. If this pathway is indeed utilized, perhaps this simplified Pol V functions in Pol IV's place as well, or one of the NRPD2s is sufficient for Pol IV function.

Post-transcriptional gene silencing

Several types of RNA silencing pathways fall into the category of post-transcriptional gene silencing (PTGS). *C. variabilis* only encodes some of the genes involved in these pathways, but nearly all were expressed during infection. In the transacting short interfering RNA (tasiRNA) pathway (Fig. 3), *Arabidopsis* uses AGO1 loaded into the RNA-induced silencing complex (RISC) to guide the cleavage of primary tasiRNA (pri-tasiRNA). One fragment is used as a template by RDR6, which works with SGS3, to produce

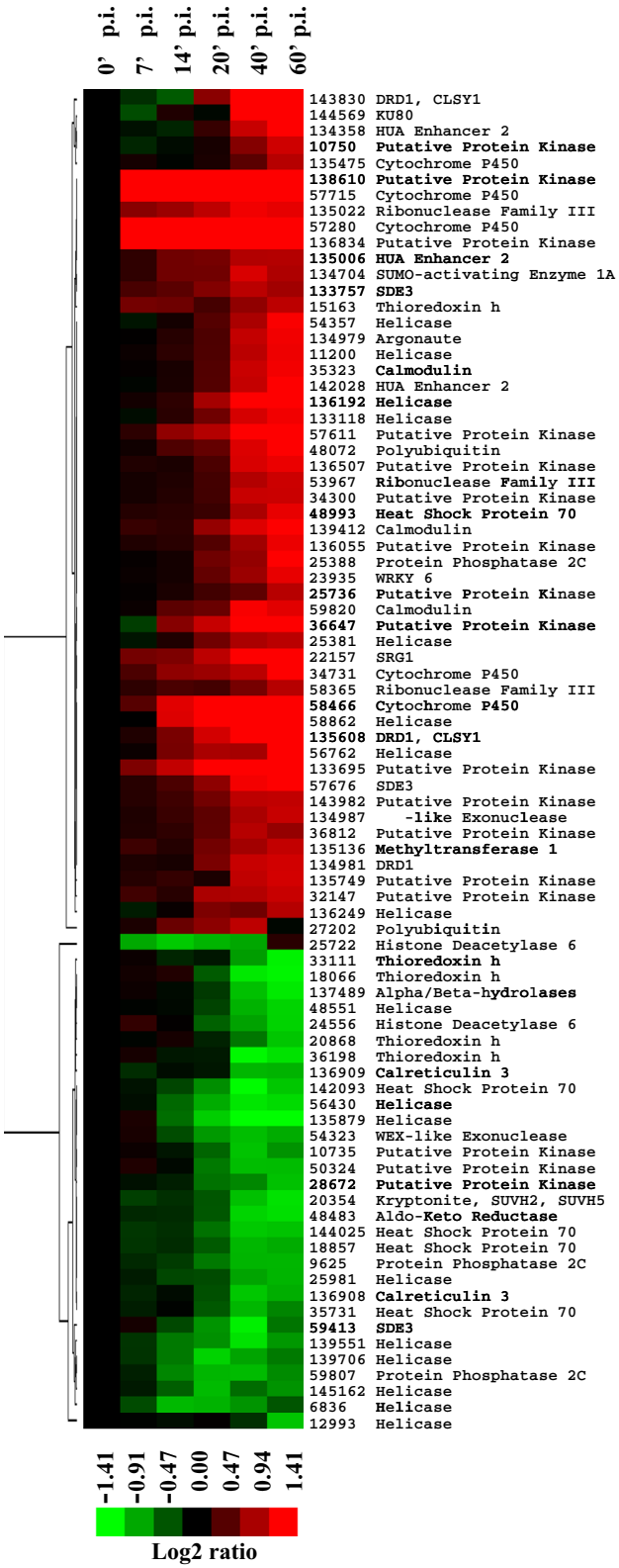

Fig. 1. Transcriptome response of 85 *C. variabilis* genes during PBCV-1 infection. Hierarchical clustering of genes having a 2-fold change for at least one time point compared with 0 min p.i.. Scale of the expression log(2) ratio changes is indicated by a color bar at the bottom. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

dsRNA, which is then processed by DCL4, and the resulting siRNAs are subsequently methylated by HEN1 (Brodersen and Voinnet, 2006). Aside from homologs to AGO1 and DCL4, no other members of this pathway were detected in the genome. In the micro RNA (miRNA) pathway (Fig. 4), pri-miRNA is processed by DCL1, HYL1, HEN1, and SE into mature, methylated miRNA. HASTY is thought to facilitate the nuclear export of miRNAs, which are then incorporated into RISC leading to translation inhibition and/or degradation via XRN4 (Brodersen and Voinnet, 2006; Park et al., 2005). Healthy and infected *C. variabilis* expressed both SE and HASTY (one homolog each) and both of its XRN4 homologs. Homologs for HYL1 or HEN1 were not detected in the genome.

XRN4 is a 5' to 3' exoribonuclease that works in several pathways, and degrades certain RNAs based on sequence or functional category (Gregory et al., 2008; Rymarquis et al., 2011; Souret et al., 2004). When this or any other means are not used to degrade atypical RNAs, the Sense-PTGS (S-PTGS) pathway can be triggered (Fig. 5). dsRNAs are produced by RDR6, SGS3, SDE3, and possibly WEX with the heterodimer, KU70/80. Subsequently, a DCL (possibly DCL4) works to produce siRNAs that HEN1 methylates. RDR6 can then either produce more dsRNA via transitivity or AGO1 along with RISC can use the siRNAs to guide the cleavage of targeted RNAs (Brodersen and Voinnet, 2006). Again, *C. variabilis* lacks RDR6, SGS3, and HEN1, but the remaining genes involved in this pathway are present in the genome and expressed during infection. Both XRN4s were expressed. Six out of seven SDE3 genes were expressed, two were up-regulated, and one down-regulated during infection. Both WEXs and each KU70 and KU80 homologs were expressed. One WEX and the one KU80 were up-regulated and the other WEX was down-regulated. Lastly within the PTGS category, the Inverted Repeat-PTGS (IR-PTGS) pathway (Fig. 6) employs DCLs (probably DCL3 and DCL4), HEN1, and AGO1. Though *C. variabilis* does not possess a homolog for HEN1, both its DCL and AGO1 homologs were expressed during infection, the latter being up-regulated.

Other genes of interest

Because RNA silencing is not required for living in certain organisms (Cerutti and Casas-Mollano, 2006), and because even if it is used by *C. variabilis*, it may not be triggered by virus infection, we examined other genes that respond to virus infection in *Arabidopsis*. However, their specific roles, if any, in the plant's defense is unknown. Homologs for 27 such genes were searched for in *C. variabilis* and 20 were detected. To maintain a focused analysis we limited our examination of genes outside of RNA silencing to these 27 genes, though we acknowledge that there are a number of other genes known to be involved in pathogen response and/or resistance in higher plants. Of those 219 homologs found in *C. variabilis*, 180 were expressed in both healthy and infected cells (five of these being putative orthologs). And of these 180, 31 were up-regulated and 17 were down-regulated ≥2-fold (Table 2). Some known functions of the homologs detected include: stress response, signaling, redox reactions, protein folding, and protein degradation (Table 1). With approximately 25% of the expressed genes experiencing a significant change in expression during infection it is likely that these genes may also be involved in *C. variabilis*'s response to virus infection or are targeted by PBCV-1 as part of its survival strategy.

Conclusions

To our knowledge, this is the first examination of higher plant defense genes in an alga (beyond core RNA silencing genes). That many RNA silencing genes were not only found, but expressed, suggests that some of the RNA silencing pathways may be utilized by *C. variabilis*. In particular, RNA directed DNA methylation and Sense post-transcriptional gene silencing are strong candidate

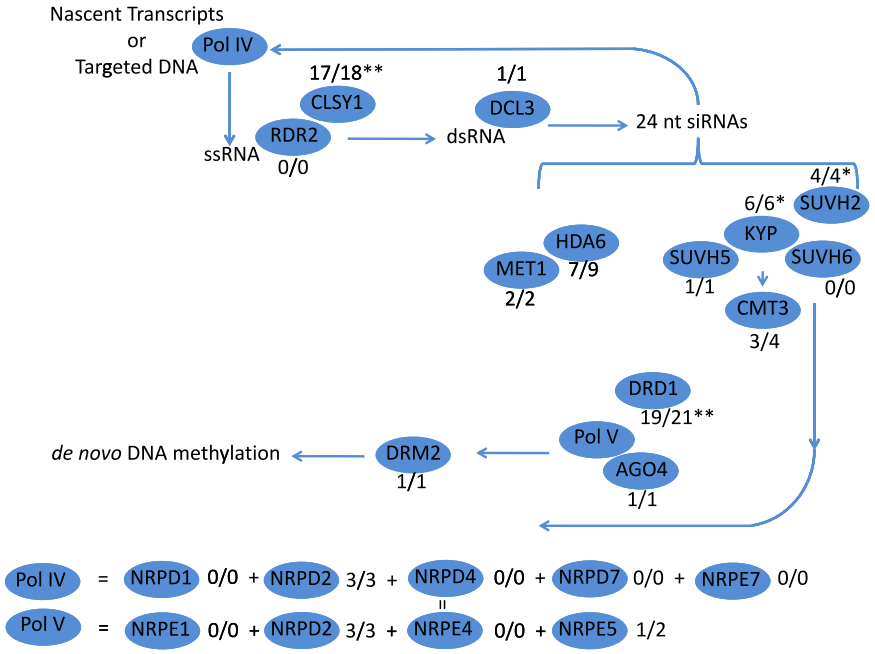

Fig. 2. Schematic of the current understanding of known proteins involved in the RNA directed DNA Methylation pathway. Numbers represent numbers of homologs expressed/numbers of homologs present in the *C. variabilis* genome. * and ** indicate shared homology.

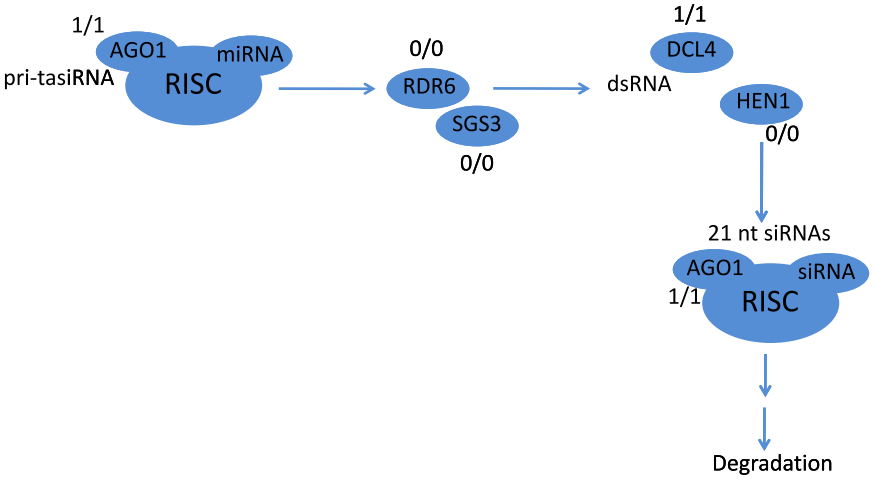

Fig. 3. Schematic of the current understanding of known proteins involved in the transacting short interfering RNA pathway. Numbers represent numbers of homologs expressed/numbers of homologs present in the *C. variabilis* genome.

pathways. Indeed RNA directed DNA methylation is an important defense strategy against DNA viruses in higher plants (Wang et al., 2012), though we admit that our DNA virus is quite different from known plant DNA viruses. We cannot definitively state that these pathways are employed by *C. variabilis* in an attempt to fend off virus infection. In its endosymbiont state, *C. variabilis*, is protected from virus infection and therefore relieved of pressure to evolve a defense mechanism. However, the finding that a quarter of the RNA silencing genes showed significantly altered expression during infection, suggests that they may be utilized for this purpose. The absence of certain homologs, in the *C. variabilis* genome, does not necessarily mean the true absence of a protein serving their function. Homologs to most of these same proteins were absent from all currently sequenced green algae. The seemingly vacant roles could be played by proteins encoded by genes too different from those in higher plants to be detected by our means, or these roles could be filled by presently detected proteins serving more than their known functions. The same can be suggested for the additional, potential defense genes we examined in this study.

The majority of genes known to be induced by virus infection in *Arabidopsis* were also expressed by *C. variabilis*, but only 31 out of 180 were up-regulated by at least a 2-fold expression ratio. These genes may serve similar virus-defense functions. Additionally, these results point to differences between green algae and land plants. With less than a quarter up-regulated during infection, one possible conclusion is that land plants have more sophisticated responses than green algae. Another possibility is that the virus is able to overwhelm the host before a successful defense using this/these means can be mounted.

Though PBCV-1 still produced a successful infection, our analyses have narrowed the list of genes possibly involved in virus

defense and have highlighted possible mechanisms not only for defense, but also for the host cell's routine regulation of gene expression. In regards to defense strategies, it is likely that green algae share more in common with higher land plants than RNA silencing alone. These studies provide additional evidence that the further examination of green algae using *Arabidopsis* as a model can be reasonable and productive. Moreover, these evaluations provide a foundation for future comparisons between virus defense mechanisms and those present in the Plant Kingdom.

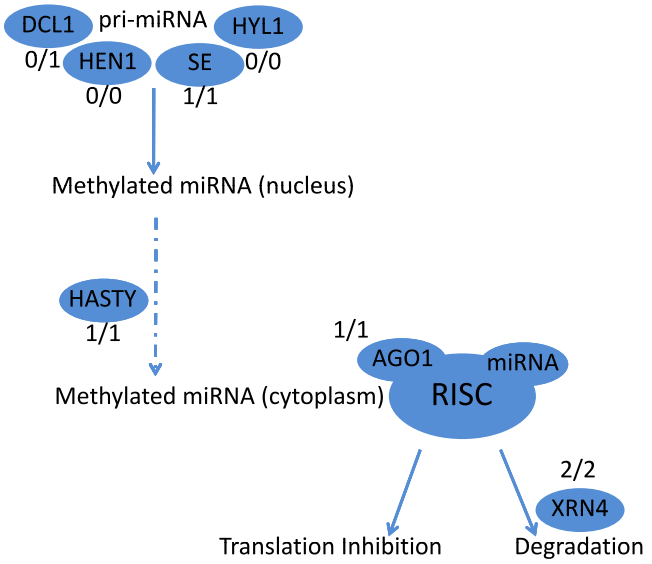

Fig. 4. Schematic of the current understanding of known proteins involved in the micro RNA pathway. Numbers represent numbers of homologs expressed/numbers of homologs present in the *C. variabilis* genome.

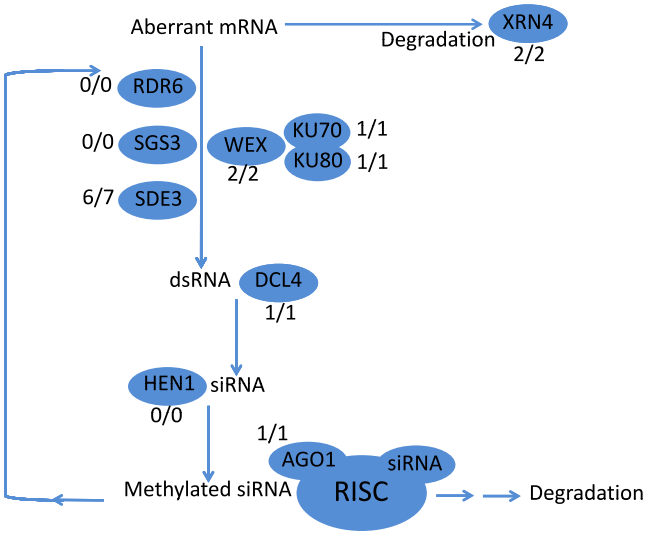

Fig. 5. Schematic of the current understanding of known proteins involved in the Sense Post-Transcriptional Gene Silencing pathway. Numbers represent numbers of homologs expressed/numbers of homologs present in the *C. variabilis* genome.

Materials and methods

Strains and culture conditions

The growth of PBCV-1 host *C. variabilis* NC64A on MBBM medium and the production and purification of PBCV-1 have been described (Van Etten et al., 1983). 3×10^9 exponentially growing cells were pelleted by centrifugation after exposure to PBCV-1 (MOI=5). Cells were immediately flash frozen in liquid nitrogen and stored at −80 °C until further processing. The durations of infection lasted for 7, 14, 20, 40, and 60 min. Cells for the 0 min. p.i. time point were not exposed to PBCV-1 until after flash freezing.

Isolation and sequencing of RNA from uninfected and PBCV-1 infected C. variabilis

The frozen *C. variabilis* cells were re-suspended in 3 ml of Trizol reagent (Invitrogen). From each sample, three aliquots, each 0.3 ml were collected from the above Trizol-cell suspension, mixed with additional 0.7 ml Trizol reagent. Total RNA was then extracted following the standard Trizol/RNeasy column procedure (Qiagen) and pooled as one sample. RNA qualities were assessed using an Agilent BioAnalyzer 2100 system (Agilent Technologies).

The RNA-seq library was constructed from 10 μg of total RNA extracted for each time point using the mRNA-seq Sample Preparation Kit (RS-100–0801) according to the manufacturer's instructions (Illumina). RNA was subjected to poly(A) selection using Sera-Mag Magnetic Oligo-dT Beads followed by fragmentation and then used for cDNA synthesis with random hexamers. The cDNA product then underwent end repair, A-tailing, adapter ligation, and PCR amplification. Each library was sequenced using an Illumina GAIIx sequencer on one lane of the flow cell, generating 15.8–19.7 million 51-nt single-end reads for each time point.

Transcriptome analysis

Reads were aligned simultaneously onto the *C. variabilis* and PBCV-1 genomes using BOWTIE2 (Langmead and Salzberg, 2012), and TOPHAT2 (Trapnell et al., 2009) for aligning reads spanning exon junctions. Only alignments that had no more than two mismatches with the reference sequence were retained. When a read produced more than one valid alignment, the genomic region producing the best alignment score was considered as its point of origin. Reads producing more than one alignment with identical best scores were considered as originating inside a repeated sequence and were discarded in subsequent per-gene read count analyses. For each time point we mapped 8.6–13.0 million reads to single copy sequences on the *C. variabilis* genome.

Of the 9776 host predicted genes, 1867 had raw read counts <50 in all time points and were discarded from subsequent analysis. The remaining 7909 genes had their read counts normalized for library size using the DESeq method (Anders and Huber, 2010). Clustering analysis and visualization of the results was done using the CLUSTER and TREE-VIEW programs (Eisen et al., 1998).

Fig. 6. Schematic of the current understanding of known proteins involved in the Inverted Repeat Post-Transcriptional Gene Silencing pathway. Numbers represent numbers of homologs expressed/numbers of homologs present in the *C. variabilis* genome.

Defense gene selection

Amino acid sequences of RNA silencing genes in *Arabidopsis* (Brodersen and Voinnet, 2006; Matzke et al., 2009) were obtained from NCBI and TAIR databases and used as queries in BLASTp searches against JGI's Model Proteins of *C. variabilis* (Altschul et al., 1990). In a similar manner, amino acid sequences of genes induced by both negative and positive sense RNA viruses in *Arabidopsis* (Whitham et al., 2006) were also used as query sequences. An alignment e-value of $1e^{-5}$ was set as the cutoff value. Helicases were added to the list of genes of interest and were found by searching the *C. variabilis* genome annotation. Focus was placed on the presence of required domains and the "probable function" of the protein in question annotated as helicase activity. Additionally, the list of protein kinase homologs was supplemented with protein kinases found in the same manner. For those proteins to which no homologs could be found, the MEME Suite (http://meme.sdsc.edu/meme/intro.html) was used to generate motifs based on sequences from other eukaryotes and also higher plants (multiple sets of MEME-based motifs/queries were generated) (Bailey et al., 2009). Detected homologs were classified based on the query sequences used to find them with the exception of the DCL homologs. Most of the DCL homologs detected lack several of the domains possessed by typical dicers. Therefore they were reclassified with the more broad description of ribonuclease family III members leaving the description of DCL to indicate the presence of all expected domains.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.04.018.

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